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<b>(54) Title:</b> A METHOD FOR PRODUCING TAXOL AND TAXANES FROM EMBRYO CULTURES OF TAXUS SPECIES  <b>(57) Abstract</b>  Disclosed herein are methods of taxol and taxane production through extraction of tissue of <i>Taxus</i> species and cell culture thereof. The method of production of taxol and its derivatives by extracting tissues and/or culture medium of <i>Taxus</i> species is characterized in that said tissue is zygote embryo. Cell culture of zygote embryo of <i>Taxus</i> species can be carried out by inducing somatic embryo or embryogenic callus.		

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## A METHOD FOR PRODUCING TAXOL AND TAXANES FROM EMBRYO CULTURES OF TAXUS SPECIES

### 5 BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention is related to a method for producing taxol and its derivatives from somatic embryos and/or culture medium, and particularly is related to a method for culturing the zygote embryo or  
10 somatic embryo to obtain somatic poly-embryos or embryogenic calli.

#### 2. Description of Prior Art

Taxol is an alkaloid which has been isolated from the plants belonging to the genus *Taxus* and exhibits significant antitumor activity  
15 in a variety of cancer cell lines, including B16 melanoma. It has been known that taxol is contained primarily in barks of *Taxus brevifolia* with approximately 0.02% by gram dry weight.

Although total synthesis of taxol has been successfully demonstrated, this method seems to be unsuitable for commercial scale  
20 production of taxol due to high cost requirement. In addition, semi-synthesis of taxol like compound (for example taxotere) also was succeeded by using baccatin III or 10-deacetyl baccatin III. However, this approach have been faced same obstacles just mentioned above.

Therefore, taxol can only be obtained from the scarce natural  
25 resources, for example raw materials of harvested yew. Because the content of taxol in yew trees is very low, 2000 to 4000 trees must cut

down to yield 1kg of taxol and production of taxol from the natural sources for sufficient demands will rise significant destruction of forest eco-system. Accordingly, there have been attempts to produce taxol and related compounds from cells and tissue cultures of *Taxus* species.

5       For example, USP 5,019,504 discloses a method for producing taxol or taxol-like alkaloids by culturing bark, cambium, needle, and root tissues of *Taxus* species in a nutrient medium to produce callus or suspension cell cultures, then recovering taxol or taxol-like alkaloids from them. However, the above method can produce only 1.0-3.0mg  
10 of taxol from 1 liter of cell suspension culture medium. The above patent teaches that bark fo Yew tree can produce optimum yield of taxol directly, and produces taxol by culturing these tissues to induce undifferentiated cell mass(callus) and/or by establishing cell suspension cultures from proliferated callus.

15       Still now, the above method using various parts of Yew tissues have not commercialized partly due to low taxol yield capacity of the cells associated or insufficient processes developed for large scale production of target secondary metabolites.

WO92-13961 discloses a method for producing taxol by culturing  
20 a tissue of a plant belonging to the genus *Taxus*, particularly its female gametophyte and recovering taxol from calli or suspension cultured cells originated from it. This process can give 0.05% of taxol based on the dry weight of suspension culture cells.

These methods fail to produce a sufficient amount of taxol in an  
25 industrial scale and till now are under development to improve the taxol production capacity. These fact suggesting that there is a need to

provide an improved method for increasing taxol contents in cell and/or culture medium and for screening high taxol producing cell line to meet final goal of industrial scale production

Under these circumstances, the present inventors have made an extensive study to develop a method for producing taxol in an industrial scale, and as a result thereof, we unexpectedly found for the first time that zygote embryo contains much larger amount of taxol, based on the same dry weight, than other tissues reported to contain taxol so far.

The present inventors have made further researches to provide a method for increasing the mass of embryo because the size of embryo itself is too small to develop an industrial scale process for producing taxol. As a result thereof, we suprisingly found that the mass of embryo can be increased to several hundred thousands times than the original size when the embryo is cultured onto nutrient media to induce a somatic embryo or embryogenic callus.

The present invention is accomplished based on the above findings.

### SUMMARY OF THE INVENTION

Thus, an object of the invention is to provide a method of production of taxol by extracting tissues of *Taxus* species, characterized in that said tissue is zygote embryo.

Other object of the invention is to provide a method of production of taxol or its derivatives by culturing a tissue of *Taxus* species and recovering taxol or its derivatives from callus or culture medium characterized in that said tissue is zygote embryo.

Still another object of the invention is to provide a method of production of taxol or its derivatives, wherein it comprises the steps of:

(a) providing living zygote embryo from *Taxus* species and disinfecting it;

5 (b) culturing said disinfected embryo onto a culture medium to produce callus from embryo;

(c) culturing the callus obtained in (b) to produce somatic embryo from said callus;

10 (d) culturing the disinfected embryo in (a) or the somatic embryo obtained in (c) to produce embryogenic callus;

(e) liquid culturing the somatic embryo in (c) or embryogenic callus in (d); and

(f) recovering taxol or taxol derivatives from the culture medium and from the cells.

15 The instant invention is distinguished from the above-cited prior arts in that it is based on the finding that the zygote embryo contains unexpectedly large amount of taxol and further increases the mass of embryo to several hundred thousand times through an induction of somatic embryo or embryogenic callus, and is advantageous over the  
20 prior art because it makes it possible to produce taxol in an industrial scale by liquid culturing the somatic embryo or embryogenic callus.

Other features and applications of the present invention will be easily apparent to the skilled in the art by the following description.

## 25 BRIEF DESCRIPTION OF DRAWINGS

Fig 1(A) is a HPLC chromatogram of standard taxanes.

Fig 1(B) is a HPLC chromatogram of purified culture medium obtained from Example 7.

Fig 2(A) is a photograph showing rat cancer cell without treatment in Experimental Example 1.

5 Fig 2(B) is a photograph showing rat cancer cell treated with the extract of Example 7 in Experimental Example 1.

Fig 3(A) is a standard curve by ELISA data.

Fig 3(B) is a curve by ELISA data carried out in Experimental Example 2.

10 Fig 4 is a graph showing effects of production medium on taxol production in Example 8.

#### DETAILED DESCRIPTION OF THE INVENTION

The present inventors analyzed taxol contents of 24,000 *Taxus*  
15 *cuspidata* trees planted in national forest land in Korea. Although the amounts were significantly varied depending on the individual tree, they found that

5g of taxol was contained in 100g of dried embryos collected from superior trees. Whereas 4.5mg and 2.0mg of taxol were obtained  
20 from the same amounts of dried bark and needle, respectively.

Therefore, the first object of the invention is to provide a method for producing taxol wherein taxol is extracted from embryo of *Taxus* species. The kind of *Taxus* species is not limited and any trees can be employed so long as it belongs to genus *Taxus*. Nevertheless, *Taxus*  
25 *cuspidata* is advantageously employed.

The extraction method for producing taxol from embryo is not

particularly limited. However, in general, extraction of taxol from embryo may be carried out by immersing freeze-dried powders of embryo in an alcohol, particularly methanol.

Unfortunately, the size of zygote embryo is very small and embryo contains more than 90% of water. Huge amount of embryos are required to produce only small amount of taxol and the extraction of taxol directly from embryo is disadvantageous in an economic viewpoint.

Accordingly, the present inventors had been made extensive researches to provide a method for producing taxol in an economic way without the above-identified problems, and as a result thereof could increase the mass of embryo to hundred thousands times by using a tissue culture techniques, particularly via an induction of somatic embryo and embryogenic callus. Therefore, the second object of the invention is to provide a method for producing taxol or taxol derivatives by inducing somatic embryo or embryogenic callus from the zygote embryo of *Taxus* species, culturing said somatic embryo or embryogenic callus in a liquid media by using a shaker or bio-reactor and recovering taxol from the culture medium and cells.

One characteristic feature of the instant invention is that the present invention can increase the mass of embryo from ten thousands to hundred thousands times through an induction of somatic embryo or embryogenic callus, whereas a simple callus induction from various tissues except embryo is difficult to attain such an amplification possibly due to the different capacities for cell division by the meristem used. Particularly, an induction of embryogenic callus

makes it possible to increase the mass of embryo to hundred thousands times and thus is very important.

The induction of somatic embryo or embryogenic callus from zygote embryo and production of taxol or its derivatives will be described hereinafter.

In order to aseptically culture an embryo, the mature or immature seeds collected from *Taxus* species during the period from August to November should be disinfected or surface sterilized. Disinfection of surface sterilization may be carried out by using common techniques. For example, seeds are immersed in 70% ethanol for 30-60 seconds, washed with sterilized water two or three times and surface sterilized with 1-3%(v/v) solution of sodium hypochlorite for 24 hours. The surface sterilized seeds are rinsed with sterilized water for more than 5 times and embryos are detached therefrom.

The embryos may be employed for extracting taxol by following the above described extraction techniques.

The embryos also are placed onto a solid nutrient medium to induce callus therefrom. Induction of somatic embryo may be carried out by PEDC(Pre-Embryogenic Determined Cell) or IEDC(Induced Embryogenic Determined Cell) procedures.

Induction of somatic embryo using PEDC pattern is as follows:

Embryo is cultured on a solid nutrient medium supplemented with 1-naphtalene acetic acid(hereinafter "NAA"), kinetin and 2,4-D for callus induction. Once callus was induced, small pieces of calli could be proliferated by subculturing onto the media contained proper concentrations of macro-, micro-nutrients, and vitamins.

For the purposes of induction and proliferation of callus, common plant tissue culture media may be employed without limitation. For example, mB<sub>5</sub>(modified Gamborg's B<sub>5</sub> medium), Durzan, MS(Murashige & Skoog medium), WPM(Lloyd & McCown),  
5 DKW(Driver-Kuniyuk-Walnut), GD(Gresshoff & Doy), SH(Schenk & Hildebrandt medium) or LP(Quoirin & Lepiover) may be employed.

It is understood that modifications may be made in these media such as addition or deletion of various components, or alteration of proportions. Among others, mB<sub>5</sub> or Durzan medium is preferred. As  
10 an intermediate step for somatic embryogenesis, the media for induction which may be the same or different from the media for rapid growth of callus may be used. From the viewpoint that the largest number of somatic embryos can be obtained, mB<sub>5</sub> medium is advantageously employed.

15 Because most of the above mentioned media are commercially available or well established, public may easily have access thereto. Therefore determination and/or optimization of suitable culture media for induction and rapid growth of callus are within the ability of a person skilled in the art. For example, mB<sub>5</sub> medium, which is  
20 advantageously employed in the invention, is slightly modified medium of Gamborg's B<sub>5</sub> and its composition is shown in Table 1.

Table 1

Compound		mg/l	Compound		mg/l
5	KNO <sub>3</sub>	2500	CoCl <sub>2</sub> .6H <sub>2</sub> O		0.025
	KCl	150	Na <sub>2</sub> MoO <sub>4</sub> .5H <sub>2</sub> O		0.25
	CaCl <sub>2</sub>	150	CuSO <sub>4</sub> .5H <sub>2</sub> O		0.025
	KI	1	H <sub>3</sub> BO <sub>3</sub>		3
	NaH <sub>2</sub> PO <sub>4</sub>	300	Thiamine.HCl		2
	MgSO <sub>4</sub> .7H <sub>2</sub> O	250	Pyridoxine.HCl		1
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	150	Nicotinic acid		1
	MnSO <sub>4</sub> .4H <sub>2</sub> O	10	Myo-innocitol		100
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	2	Sucrose		30000
	Na <sub>2</sub> -EDTA	37.2			
10	FeSO <sub>4</sub> .7H <sub>2</sub> O	27.8	pH		5.6-5.7

Besides, IEDC pattern for the production of somatic embryo may be carried out by culturing embryo in an above-illustrated medium, to which different plant growth hormones are added alternatively.

For the initial culutre, 2-4 ppm of 2,4-D is added. When callus is formed from the surface of embryo and the cell mass increases 10-20 folds, subculture was usually followed by using the same medium having half concentration of the auxius used.

Because most of the callus lose their growth ability due to the phenolic compounds possibly secreted from the non-prolific cells, 0.5%(w/v) of activated charcol or 1-2%(w/v) ployvinly polypyrrolidine were incorporated. The callus is subcultured onto a medium free of growth regulator for 2-4 months to form somatic embryo from meri- stemic tissues of callus.

Thus-formed somatic embryo contains large amount of taxol or

taxol derivatives(hereinafter, "taxanes") and may be employed per se to extract taxanes. The extraction of taxanes from somatic embryos may be carried out by using common methods, particularly the method described hereinafter. However, the somatic embryo is  
5 advantageously employed to induce embryogenic callus in order to further increase cell mass.

According to the present invention, a relatively high rate of taxanes can be obtained by extraction of embryogenic calli as well as the above produced somatic embryos. The embryogenic callus may  
10 be produced by culturing the somatic embryo obtained by PEDC or IEDC procedure described as above or the zygote embryo. Procedures for the production of embryogenic callus is as follows: The disinfected embryo or somatic embryo is cultured for 2-4 months in a solid medium suitable for inducing callus, which is supplemented 1.0-4.0  
15 ppm of NAA and 0.5-2.0 ppm of kinetine, preferably with 2.0 ppm of NAA and 1.0 ppm of kinetin. 1-4 ppm of 2,4-D instead of NAA plus kinetin may be added to the medium. The induced callus is then cultured onto the same medium supplemented with 1-5 ppm of cytokinin such as benzylamine purine, N-isopenthenyl aminopurine,  
20 kinetin or zeatin for 1-2 months to form embryoid on the surface of the callus, and the embryoid is cultured onto the same medium supplemented with 2-10 ppm of 2,4-D at about 26°C for 2-3 months under the 16/8 hours(light/dark) condition. This culture enables to obtain embryogenic callus in a huge amount which is hundred  
25 thousands times of original mass of embryos. The embryogenic callus showed a wide range of color, for example, from yellow, brown to

green.

For the induction and proliferation of embryogenic callus, any medium which can be employed for conventional plant cell and tissue cultures may be used without limitation.

- 5       The above-produced embryogenic callus contains a large amount of taxol and may directly be employed for extraction of taxol and taxanes.

The extraction of embryogenic callus may be carried out by a common technique, particularly the method described hereinafter.

- 10       In order to amplify the mass of the embryogenic callus in an industrial scale, the embryogenic callus is aseptically sliced to produce single cells or small cell aggregates. To establish cell suspension cultures, 10%(v/v) of these cells were inoculated into 250ml conic flask having 50ml of a liquid culture medium supplemented with 2ppm of  
15 2,4-D. Air-lift or impeller type bioreactors are advantageously employed for this purpose in the viewpoint of continuous culture.

- The bioreactor culture may be carried out using two different culture media: one for the growth of cells(Growth medium) and the other for the production of taxanes(Production medium). The growth  
20 medium is preferably mB<sub>5</sub> medium supplemented with 2-4 ppm of 2,4-D and the production medium is preferably MS medium supplemented with 2-10 ppm of NAA.

- Particularly, the production medium preferably contains elicitors in order to increase the taxol production. The elicitors may be  
25 selected from fungal elicitors, female gametophyte extract(1-5 ml/l), phenylalanine(50-300 mM) and GA3(0.5-1.0 ppm). The examples of

5 fungal elicitors include *Cytospora abietis* ATCC No. 38688 and *Penicillium minioluteum*(Dierckx), NRRL 18467, which are pulverized and subjected to a series of extraction to give extracts. Alternatively, it is also preferable to collect and culture fungi of *pestalotiopsis* genus and to add it's extract to the culture medium in an amount of 0.5-1.0 ml/l.

10 Taxol is present in cells as well as secreted into the culture medium. Accordingly, after the liquid culture process, taxol and taxanes can be recovered from the cultures or the media by using well known techniques. Separation of cells from the culture medium may be carried out by centrifugation(for example 1000xg, 5-10 minutes) or by decantation. If the later technique is employed for the separation, the culture broth is allowed to stand for about 24 hours to precipitate cells and the culture medium is decanted. The culture medium still  
15 remained in the collected cells is removed by vacuum suction using pasteur pipette.

Taxanes could be recovered from the somatic embryo or embryogenic callus cells as well as the culture medium by extracting them with alcohols or a 1:1 mixture of methanol and methylene  
20 chloride. The precipitates formed during centrifugation may be pulverized by using a sonicator( Branson 250) and subjected to extraction.

According to the present invention, taxol itself as well as various taxol derivatives are recovered. Taxol derivatives include 10-deacetyl  
25 baccatin III, baccatin III, 10-deacetyl taxol, cephanolmannin and 7-epi-10-deacetyl taxol(see Fig 1(B)). The taxol derivatives may be used as

such or converted to taxol by semi-synthesis, if necessary.

The identification of taxanes from the somatic embryo and embryogenic callus originated from zygote embryo was accomplished by HPLC, cytotoxicity test and ELISA using taxol and six(6) taxol  
5 derivatives supplied from NCI as standards.

The present invention will be described in more detail by way of non-limiting Examples.

#### Example 1

Taxol was recovered from the zygote embryo of *Taxus* species.  
10 Seeds were harvested from superior trees of *Taxus cuspidata*.  
The embryo was dried in a vacuum oven of about 40°C for about 3 days to water content of less than 10% and weighed. Then, embryo was freezed with liquid nitrogen and pulverized. Pulverized embryo was immersed in an incubator of about 28°C containing about 100  
15 weight times methanol for about 7 days. The resulting extract was filtered through a membrane filter(pore size, 0.2μm) and analyzed for the content of taxol by using HPLC.

Bark and needles collected from the same tree were treated and analyzed for the content of taxol by following the same procedure as  
20 described above.

The results are shown in Table 2.

Table 2

Tissue	Taxol content in the dry tissue(100g)
Embryo	5g
Bark	4.5mg
Needle	2.0mg

Example 2

Somatic embryo was produced from the embryo by using PEDC  
10 pattern.

Seeds from selected genotype of *Taxus cuspidata* were surface  
sterilized by immersing them into 70% ethanol and 2% sodium  
hypochlorite for 50 seconds and 24 hours respectively, and rinsing with  
sterile distilled water for 3 to 5 times after the end of each step.

15 As a culture medium, mB<sub>5</sub> medium (having the ingredients and  
compositions shown in the above Table 1) supplemented with 2.0ppm  
NAA, 0.5ppm kinetin and 0.5ppm 2,4-D was employed. *In vitro*  
culture was carried out in a growth room adjusted to 26°C for 8 weeks.

During the first 8 weeks of culture, the embryos enlarged and  
20 callus was induced from surface of the most embryos. The green  
spots in the callus were excised and cultured in mB<sub>5</sub> medium without  
any plant growth regulators for about 3 months to induce somatic  
embryos.

Example 3

25 Somatic embryo was produced from the zygote embryo by using  
IEDC pattern.

Seeds from *Taxus cuspidata* were sterilized by following the procedure in Example 2 and embryos were isolated therefrom. Embryos were cultured in Durzan medium supplemented with 2ppm 2,4-D at 23 - 28°C for 2 weeks. After 2 weeks of culture, all embryos were entirely covered with calli induced therefrom. After 4 weeks of culture the mass of calli reached to several ten times of the original mass.

In order to avoid any inhibition of growth or death of callus due to phenols secreted by itself, subculture of the callus was carried out by using the same medium supplemented with about 1% of polyvinyl polypyrrolidone. After 8 weeks culture, when the average diameter of the calli reaches to 1 - 1.5cm, the callus was moved to mB<sub>5</sub> medium containing no plant growth regulator, wherein callus was grown to induce somatic embryos. Differentiation of roots was observed in some of the embryos.

#### Example 4

In order to induce embryogenic callus cultures, sterilized zygote embryo in Examples 2 and somatic embryos in Examples 3 and 4 were employed as starting materials.

The above materials were cultured in mB<sub>5</sub> medium supplemented with 2.0ppm NAA and 1.0ppm kinetin at 23-28°C for about 2.5 months to induce callus. After the completion of callus induction, subculture was conducted using the same medium with 3ppm of zeatin for about 6 weeks to obtain green to yellowish embryoids.

Embryoids were cultured on the same medium containing 3ppm of 2,4-D at 26°C for about 2 months under the 16/8(light/dark) hours

condition to produce embryogenic callus which amounted to hundred thousands times of original embryo mass. The resulting embryogenic callus differs in morphology compared with normal callus originated from various type of Yew tissues and sometimes produced similar  
5 appearances of zygotic embryo.

However, the surface of embryogenic callus were consisted of various color ranging red, yellow, dark green, light brown, dark brown and black. Each colored cell mass were sliced with blade to produce small cell aggregates, which were then subcultured into liquid media  
10 for 2-4 weeks. After removal of large cell clumps from the liquid cultures, single cell and/or small cell aggregates were inoculated onto the plastic Petri dish having 20ml of culture medium. The method incorporated is liquid plating and selection of differently colored cell lines were conducted after 4 weeks of culture. These visual selection  
15 to obtain different colored cell lines were routinely conducted.

#### Example 5

Somatic embryos obtained in Example 2 or 3, or embryogenic calli obtained in Example 4 were dried at 25°C and dissolved in 1μl of methylene chloride. 1μl of distilled water was added and the  
20 mixtures were stirred for 10 seconds, followed by centrifugation(25,000 x g). The precipitates were dried at 25°C, dissolved in 50μl of methanol and filtered through 0.2μm filter to obtain extracts.

Extracts were analyzed by HPLC equipped with a reverse-phase micropore column using a standard curve(correlation coefficient 0.999)  
25 of external standards. The HPLC results showed that the peak of the standard taxanes and those of taxane contained in the above extracts

appeared at the same retention time(14.3 minutes).

#### Example 6

Somatic embryo in Examples 2 and 3, and embryogenic callus in Example 4 were extracted to produce taxanes as follows:

5 Each 0.5g of somatic embryo in Examples 2 and 3, and embryogenic callus in Example 4 were placed in a centrifugal tube and 2 $\mu$ l of hexene was added. The mixtures were well mixed using a glass rod and stored at -20°C for 12 hours, followed by centrifuging at 25°C, 25,000 x g for 20 minutes.

10 To precipitate were added a mixed solution of methanol:ethylene chloride and subjected to sonication using a sonicator(Branson 250). The resulting solutions were centrifuged at 25,000 x g and the precipitates were dried at 60°C to dryness to obtain extracts.

The extracts were analyzed for their taxol contents and the results  
15 were as follows : the extract of somatic embryo of Example 2 contained 0.21-0.27mg per g of dry cells(total taxol content was 0.5-1.2mg), the extract of somatic embryo of Example 3 contained 0.20-0.27mg(total taxol content was 0.55-1.1mg) and the extract of embryogenic callus of Example 4 contained 0.23-0.28mg(total taxol  
20 content was 0.6-1.4mg).

#### Example 7

For the mass production of embryogenic callus, embryogenic callus of Example 4 was cultured using a impellar type bioreactor.

A embryogenic callus of Example 4 was inoculated at 10%  
25 PCV(Packed Cell Volume) onto a 250ml Erlenmeyer flask having 50ml of liquid mB<sub>5</sub> medium with 2ppm of 2,4-D.

Stationary phase was attained when cultured at 25-28°C for 18 days under aseptic condition.

The cultures were placed in a 5 liter impeller type bioreactor containing MS medium supplemented with 2ppm of NAA(production  
5 medium). Maintenance of culture was carried out at 25-28°C for 30 days under an aerobic condition. After 30 days of culture, the culture broth was allowed to stand for 24 hours to precipitate cells and the cells were separated from the culture medium. Pasteur pipette was used to thoroughly remove culture medium from the cells.

10 Thus separated cells and culture medium were extracted to produce taxol and its derivatives in the same manner described in Example 5.

The HPLC results showed that the peaks of the standard taxol and its derivatives(Fig 1(A)) and those of taxol and its derivatives  
15 contained in the above extract(Fig 1(B)) appeared at the same retention time, indicating that these compounds are the same.

It was calculated that the cells contained 0.09mg of taxol per g of dried cells while the culture medium contained about 8mg of taxol/l.

#### Example 8

20 In order to examine the effect of the type of culture media on taxol production, the cultures of Example 7 were inoculated onto various culture media such as MS, mB<sub>5</sub>, WPM, DKW, Durzan, White, LP, GD, B<sub>5</sub>, DCR or SH media. All the media tested were supplemented with 2.0 ppm of NAA and other factors including micro-  
25 environmental conditions were equally adjusted. The period of culture was reduced to 20 days.

The results are shown in Fig. 4. As can be seen from Fig. 4, the production of taxol was significantly influenced by the type of the culture medium and MS and mB5 gave the highest production of taxol.

#### Wxample 9

5 Various elicitors may be added to the production medium to increase taxol and taxane production. The effect of elicitors on taxol production was evaluated as follows:

(1) For the present invention, an extract of *Pestalotiopsis* sp., which is a fungium occurred in *Taxus* species, was added to the  
10 production medium as an elicitor for taxol production.

Plant tissues such as seed and inner bark were immersed in 70% ethanol, surface-sterilized using 15% H<sub>2</sub>O<sub>2</sub> for 15 minutes and then again immersed in 70% ethanol. Tissues were rinsed with sterile distilled water for 4 times or more to remove remaining agents.

15 Thus surface-sterilized tissues were placed on media: malt extract agar medium(malt extract 20.0g, peptone 5.0g, agar 15.0g and distilled water 1 liter), growth agar medium(glucose 40g, bacto soyton 10g, sodium acetate 1g, sodium benzoate 50mg, agar 20.0g and distilled water 1 liter) and water medium(agar 20.0g and distilled water 1 liter)  
20 and maintained in a thermostatically controlled growth chamber with 12/12(dark/light) hours of elumination.

When *Pestalotiopsis* sp. fungi were appeared within 3 days of culture, the fungi were further cultivated on the malt extract and growth agar medium(yeast extract 3g, bacto soyton 5g, MgSO<sub>4</sub> 0.5g,  
25 glucose 5g, sucrose 10g/L) for 4 days. When the time of harvest, the culture broth was centrifuged to separate cells from the culture

medium. The cells were dried, pulverized and extracted with methanol to give carbohydrate fraction.

Thus obtained carbohydrate extract of *Pestalotiopsis* sp. was added to the production medium to a concentration of 1ppm, and the culture and analysis were carried out by following the same procedure in Example 7. The results are shown in Table 3.

(2) The procedure in Example 7 was repeated except that female gametophyte extract 2ml/L, phenylalanine 100mM or Gibberellic acid 1ppm was added as an elicitor to the production medium. The results are shown in Table 3.

Table 3

Elicitor	Amount of taxol	
	per liter of culture medium	per 100g of live cell
Funfi extract	30mg	6.3mg
Female gametophyte extract	25mg	5.5mg
Phenylalanine	21mg	4.1mg
Giberelline	28mg	6.0mg

#### Experimental Example 1

Verification of taxol from embryo cultures of Yew tree was accomplished by cytotoxicity test using a rat cancer cell.

Cytotoxicity test using a rat cancer cell is usually carried out based on the fact that taxol is capable of killing selectively cancer cells

at the metaphase of the cell division. Rat cancer cells provided by Central Research and Development Center of Pacific Corporation, in singal, Kyounggi-do, Korea were cultured in plastic culture vessels containing an animal cell culture medium. At the early period of culture, when cell division was occured, a drop of the extracts from Example 7 was added to one vessel(Treatment) while no extract was added to another vessel(Control). Then, cell division and viability of each cancer cell were examined. The results are shown in Fig 2(A) and Fig 2(B).

As can be seen from Fig 2(A) and 2(B), the control group showed flourishing growth of cancer cells (Fig 2(A)) while the treatment group showed the death of cancer cells(Fig 2(B)). The death of cancer cells was appeared after 3 hours from the treatment and completed about 24 hours later. Therefore, measurements of cell division or viability was not required.

#### Experimental Example 2

The identification of taxanes from embryo cultures of Yew trees was also accomplished by ELISA(enzyme line immunosorbent assay) using monoclonal antibody as follows:

TA01 kit responding specifically to taxol and TA03 kit responding specifically to taxanes, all of which were purchased from Hawaii Biotechnology Group, were employed for this experiment. Taxol- and taxane-antigens were diluted to 1/100 folds with PBS(phosphate buffered saline) and 100 l of the dilution was distributed into ELISA plate. After incubating at 25°C for 1 hour, the plate was washed with TBS-T(washing buffer) solution at least four times, and

50 $\mu$ l of PBST(phosphate buffered saline tween) was added thereto. Taxol standard, taxane standard or the extracts of Example 7 was distributed into the well in a consecutive three folds dilution manner.

Taxol-antibody and taxane-antibody were diluted with PBST to 1/100  
5 and 1/1000 folds, respectively and 50 $\mu$ l of each dilution was distributed. After incubating at 25°C for 1 hour, the plate was washed with the washing buffer 4 times. 100 $\mu$ l of HRP(horse radish peroxidase) diluted with PBST to 1/2000 folds was distributed into the well and incubated at 25°C for 1 hour, followed by washing with the  
10 washing buffer 4 times. 200 $\mu$ l of OPD(o-phenylenediamine) was added and incubated at 25°C for 1 hour to develop a color.

Absorbance at 490nm was measured by using a ELISA reader. The results are shown in Fig 3(A) for the taxol standard and in Fig 3(B) for the culture medium of Example 7, which demonstrated taxol activity of  
15 the embryo cultures of *Taxus* species. It is understood that the foregoing detailed description is given merely by way of illustration and that modification and variations may be made therein without departing from the spirit and scope of the invention.

**WHAT IS CLAIMED IS:**

1. A method of production of taxol by extracting tissues of *Taxus* species, characterized in that said tissue is zygote embryo.
2. A method of production of taxol or its derivatives by  
5 culturing a tissue of *Taxus* species and recovering taxol or its derivatives from callus or culture medium, characterized in that said tissue is zygote embryo.
3. The method as claimed in claim 2, wherein the method comprises the steps of:  
10 (a) providing living azygote embryo from seed of *Taxus* species and disinfecting it;  
(b) culturing an inoculation of said disinfected embryo onto a culture medium to produce callus from embryo;  
(c) culturing the callus obtained in (b) to produce somatic embryo  
15 from said callus;  
(d) culturing the disinfected embryo in (a) or the somatic embryo obtained in (c) to produce embryogenic callus;  
(e) liquid culturing the somatic embryo in (c) or embryogenic callus in (d); and  
20 (f) recovering taxol or taxol derivatives from the culture medium and from the cells.
4. The method as claimed in claim 3, wherein the step (c) is carried out by inducing the somatic embryo by employing PEDC(Pre-Embryogenic Determined Cell) or IEDC(Induced Embryogenic  
25 Determined Cell) procedure.
5. The method as claimed in claim 3, wherein the liquid culture

(e) is consisted of a growth stage using a growth medium and a taxol production stage using a production medium.

6. The method as claimed in claim 5, wherein the growth medium is a modified Gamborg B<sub>5</sub>(mB<sub>5</sub>) medium having 2-4 ppm of 2,4-D and the production medium is MS or mB<sub>5</sub> medium supplemented with 1-2ppm of NAA.

7. The method as claimed in claim 5, wherein the production medium contains as an elicitor a carbohydrate fraction extracts from fungi occurred in *Taxus* species, an extract of female gametophyte of *Taxus* species, phenylalanine or Gibberelline to increase taxol producing capacity.

8. The method as claimed in claim 7, wherein the fungi is *Pestalotiopsis* sp.

9. The method as claimed in claim 3, wherein the step (d) is carried out by culturing the embryo in the step(b) or the somatic embryo in the step(c) in a solid medium containing 1.0-4.0ppm of NAA and 0.5-2.0ppm of kinetin to induce a callus therefrom, culturing the callus in the same medium containing 1-5ppm of cytokinin, benzylamine purine, N-isopentenyl aminopurine, kinetin or zeatin to form embryoids on the surface of the callus and cultuing the embryoids in the same medium containing 2-10ppm of 2,4-D to produce embryogenic callus.

10. The method as claimed in claim 3, wherein the somatic embryo in the step(c) is extracted to produce taxol or its derivatives.

11. The method as claimed in claim 3, wherein the embryogenic callus in the step(d) is extracted to produce taxol or its

derivatives.

12. The method as claimed in claim 2, wherein the *Taxus* species is *Taxus cuspidata*.

13. A somatic embryo cultures from zygote embryo explants of  
5 *Taxus* species which is obtained in the step (c) of claim 3.

14. A embryogenic callus cultures from zygote embryo explants  
of *Taxus* species which is obtained in the step (d) of claim 3.

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## AMENDED CLAIMS

[received by the International Bureau on 16 November 1994 (16.11.94);  
original claims 1,13 and 14 cancelled;  
remaining claims unchanged (3 pages)]

1. (Cancelled)

2. A method of production of taxol or its derivatives by  
5 culturing a tissue of *Taxus* species and recovering taxol or its  
derivatives from callus or culture medium, characterized in that said  
tissue is zygote embryo.

3. The method as claimed in claim 2, wherein the method  
comprises the steps of:

10 (a) providing living azygote embryo from seed of *Taxus* species and  
disinfecting it;

(b) culturing an inoculation of said disinfected embryo onto a  
culture medium to produce callus from embryo;

(c) culturing the callus obtained in (b) to produce somatic embryo  
15 from said callus;

(d) culturing the disinfected embryo in (a) or the somatic embryo  
obtained in (c) to produce embryogenic callus;

(e) liquid culturing the somatic embryo in (c) or embryogenic callus  
in (d); and

20 (f) recovering taxol or taxol derivatives from the culture medium  
and from the cells.

4. The method as claimed in claim 3, wherein the step (c) is  
carried out by inducing the somatic embryo by employing PEDC(Pre-  
Embryogenic Determined Cell) or IEDC(Induced Embryogenic  
25 Determined Cell) procedure.

5. The method as claimed in claim 3, wherein the liquid culture

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(e) is consisted of a growth stage using a growth medium and a taxol production stage using a production medium.

6. The method as claimed in claim 5, wherein the growth medium is a modified Gamborg B<sub>5</sub>(mB<sub>5</sub>) medium having 2-4 ppm of 2,4-D and the production medium is MS or mB<sub>5</sub> medium supplemented with 1-2ppm of NAA.

7. The method as claimed in claim 5, wherein the production medium contains as an elicitor a carbohydrate fraction extracts from fungi occurred in *Taxus* species, an extract of female gametophyte of *Taxus* species, phenylalanine or Gibberelline to increase taxol producing capacity.

8. The method as claimed in claim 7, wherein the fungi is *Pestalotiopsis* sp.

9. The method as claimed in claim 3, wherein the step (d) is carried out by culturing the embryo in the step(b) or the somatic embryo in the step(c) in a solid medium containing 1.0-4.0ppm of NAA and 0.5-2.0ppm of kinetin to induce a callus therefrom, culturing the callus in the same medium containing 1-5ppm of cytokinin, benzylamine purine, N-isopentenyl aminopurine, kinetin or zeatin to form embryoids on the surface of the callus and cultuing the embryoids in the same medium containing 2-10ppm of 2,4-D to produce embryogenic callus.

10. The method as claimed in claim 3, wherein the somatic embryo in the step(c) is extracted to produce taxol or its derivatives.

11. The method as claimed in claim 3, wherein the embryogenic callus in the step(d) is extracted to produce taxol or its

derivatives.

12. The method as claimed in claim 2, wherein the *Taxus* species is *Taxus cuspidata*.

13. (Cancelled)

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14. (Cancelled)

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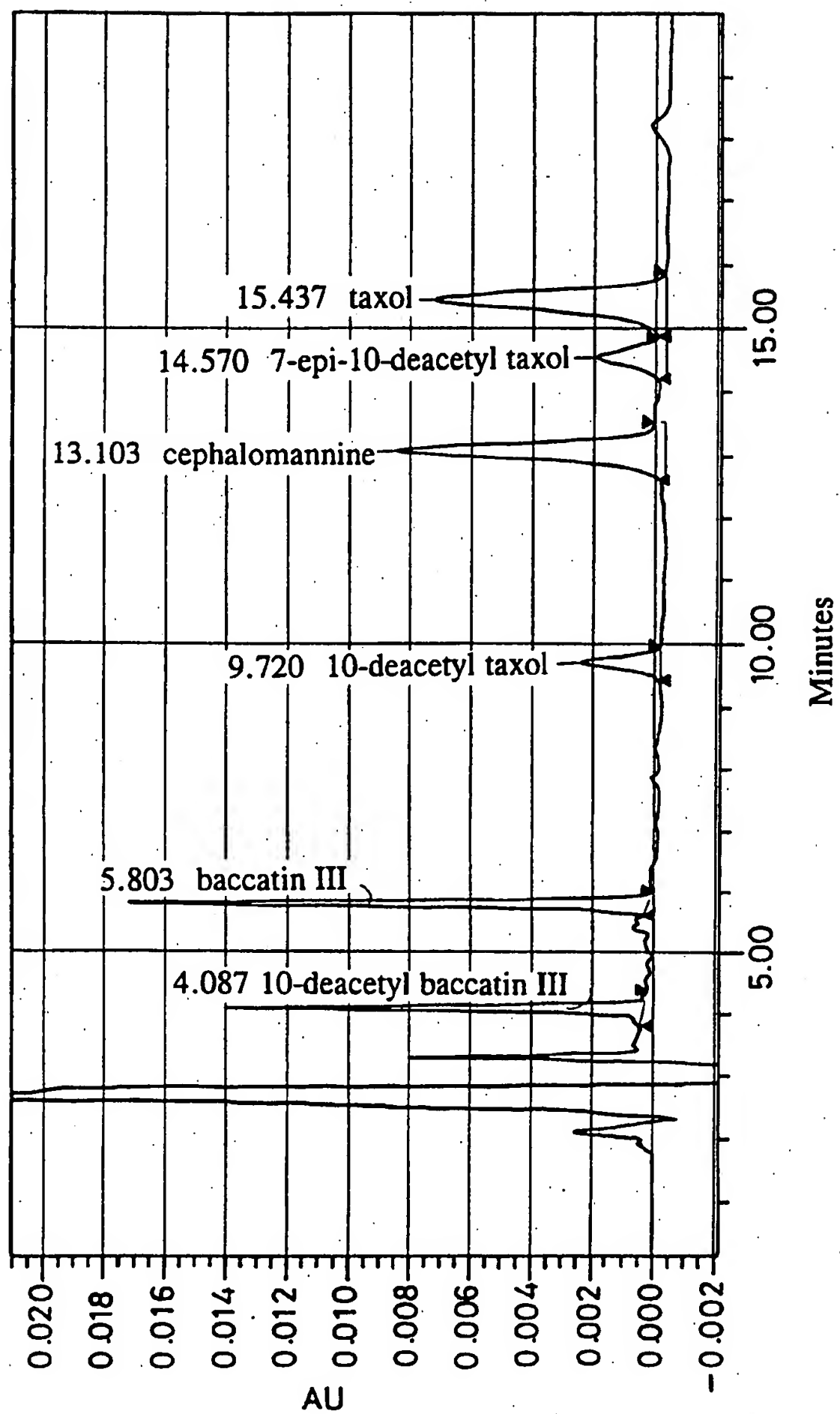
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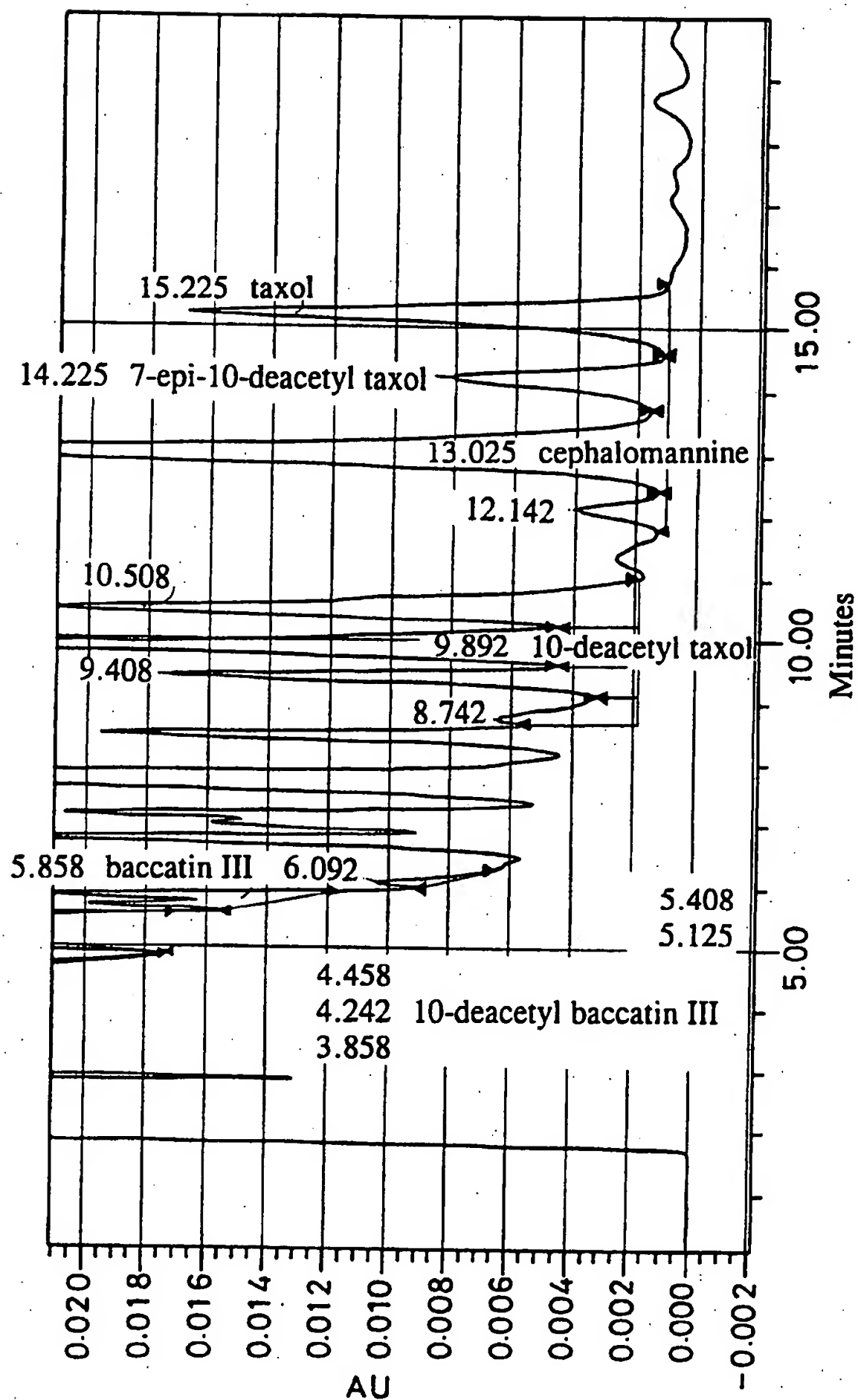
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FIG. 1(A)



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FIG. 1(B)

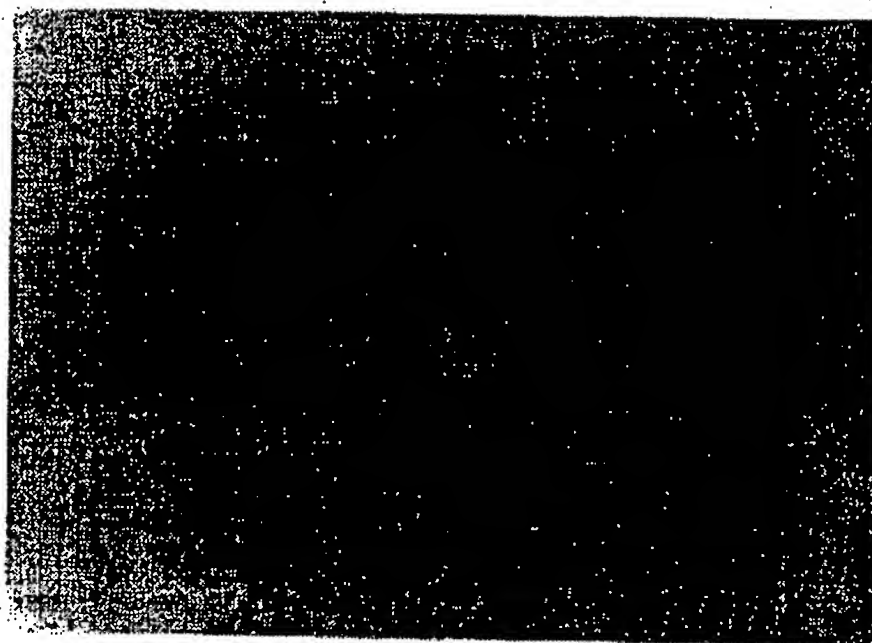


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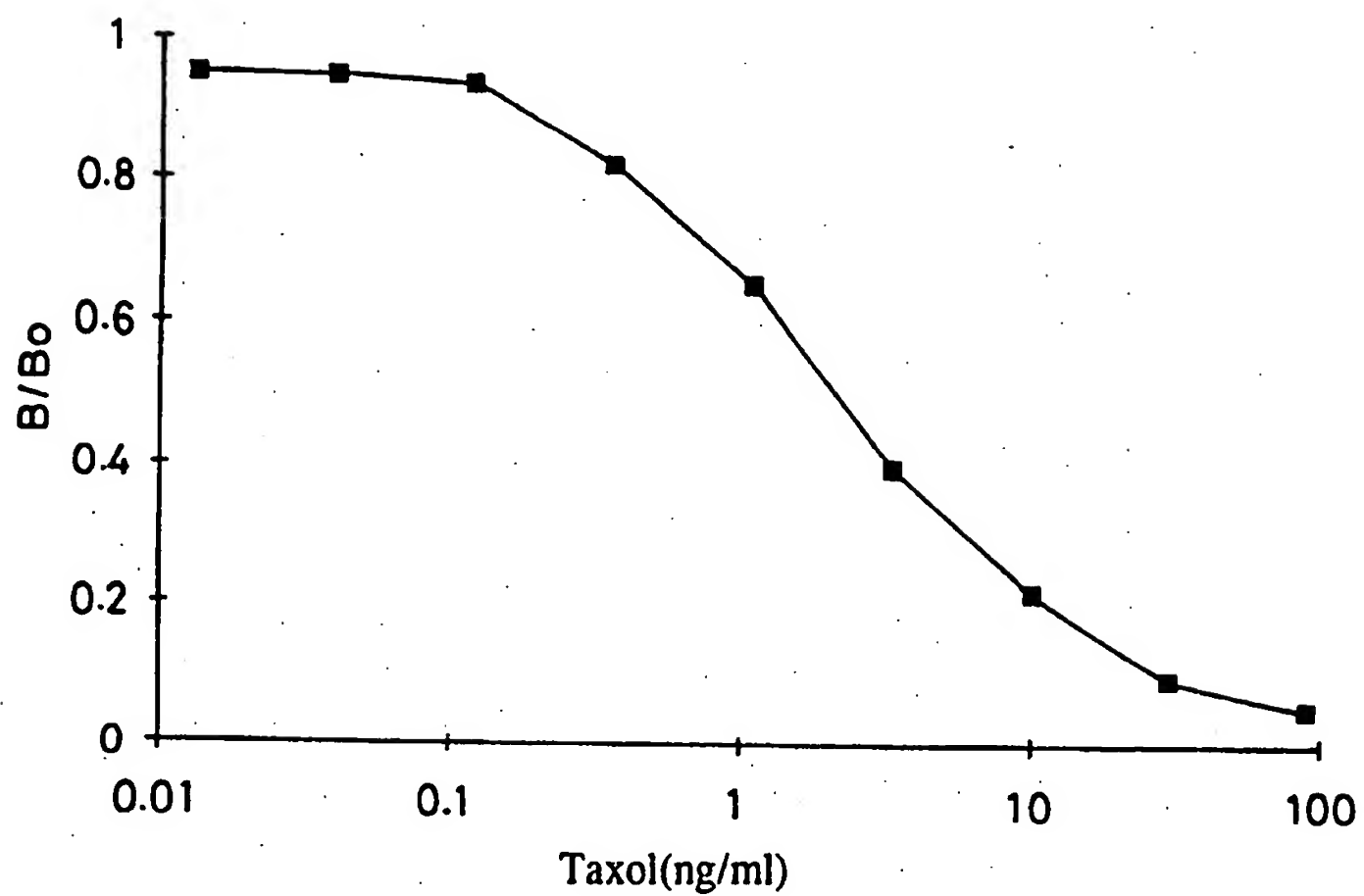
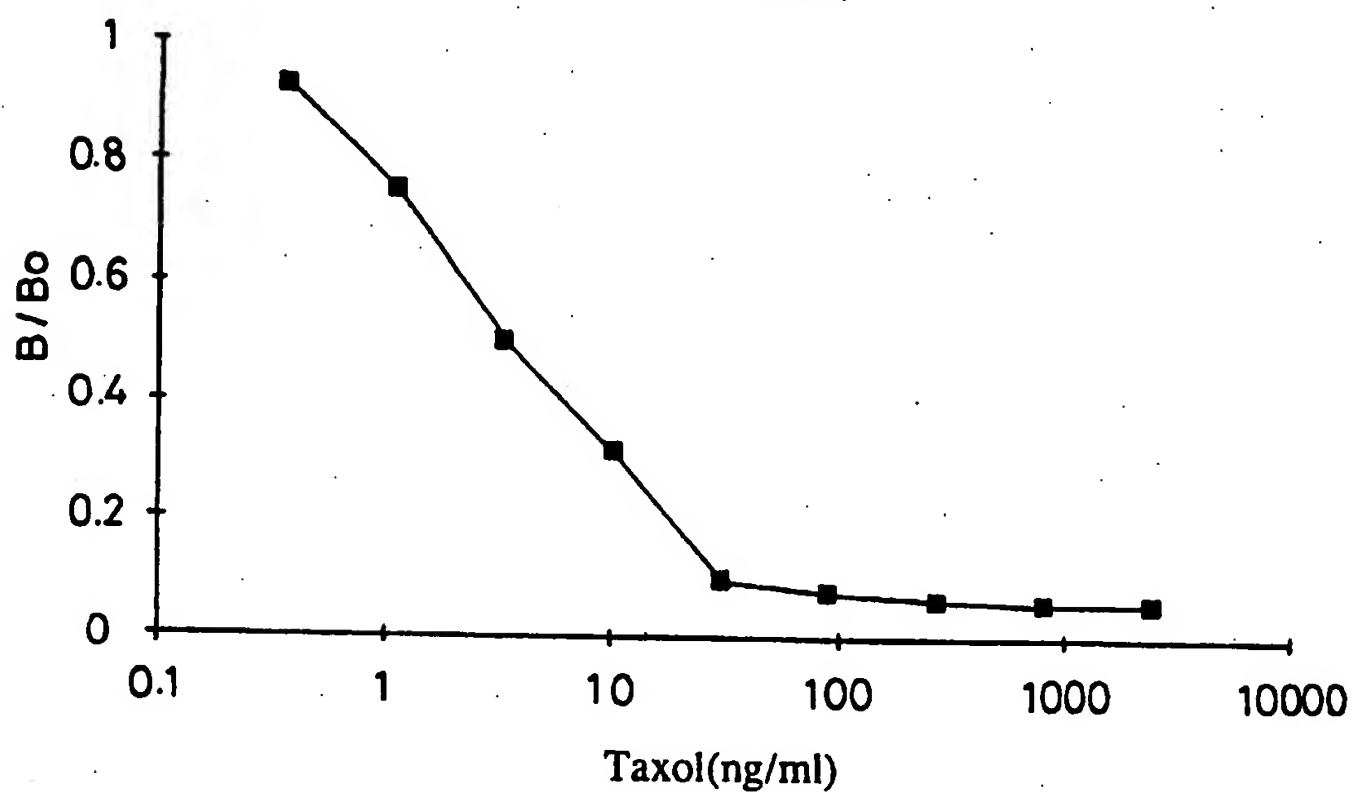
**FIG. 2(A)**



**FIG. 2(B)**

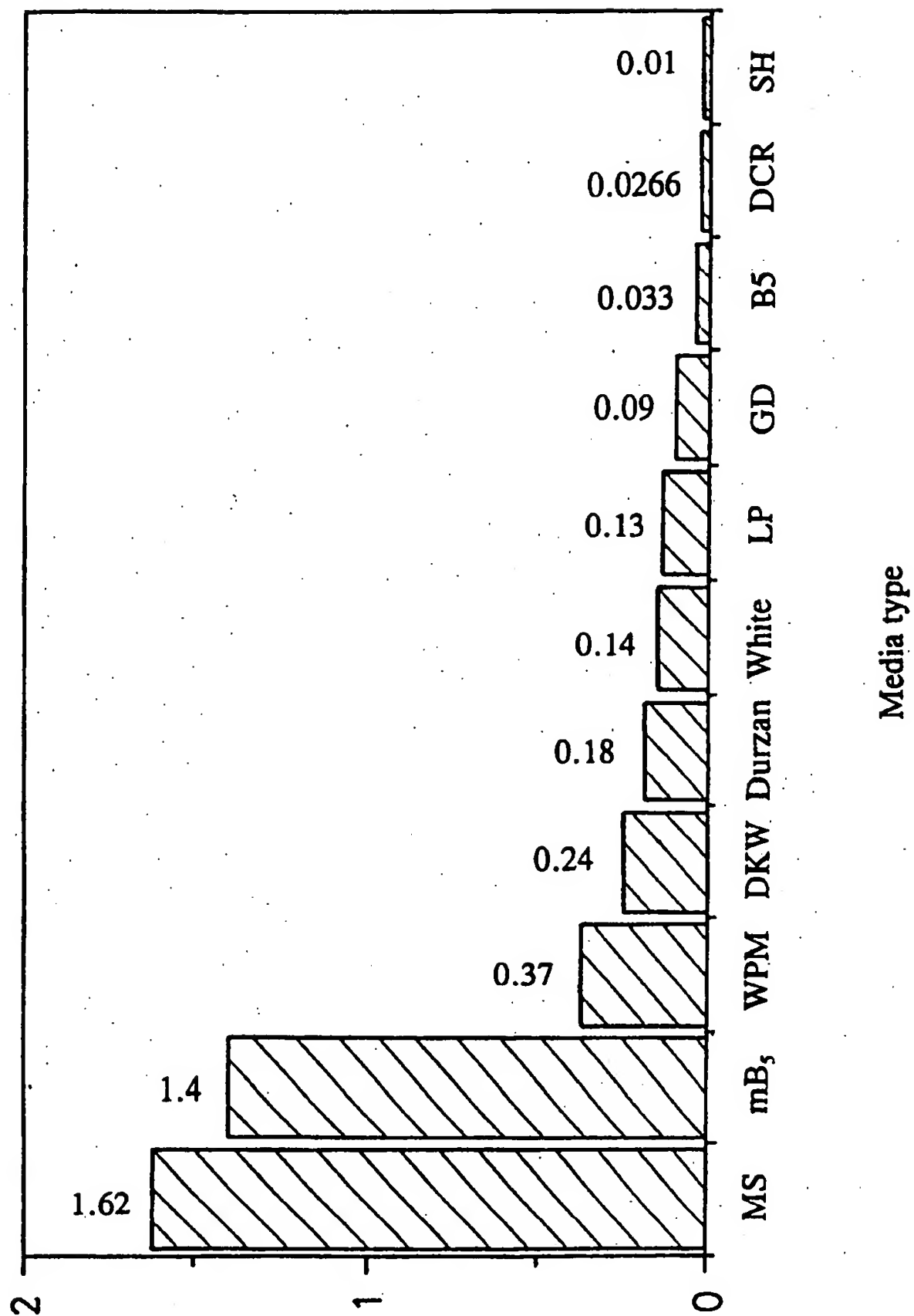


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**FIG. 3(A)****FIG. 3(B)**

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FIG. 4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 94/00088

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>6</sup>: C 12 P 17/02; C 12 N 5/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>6</sup>: C 12 P 17/02; C 12 N 5/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO, A1, 93/19 585 (UNION CAMP CORPORATION) 14 October 1993 (14.10.93), claims 1-3,12,13.	1,12-14
A,P	WO, A1, 93/23 555 (THE PENN STATE RESEARCH FOUNDATION) 25 November 1993 (25.11.93), claims 1-3.	1
A,P	WO, A1, 93/21 338 (THE RESEARCH AND DEVELOPMENT INSTITUTE) 28 October 1993 (28.10.93), claims 1,2.	1,12
A,P	WO, A1, 93/17 121 (PHYTON CATALYTIC) 02 September 1993 (02.09.93), claims 1,5.	1,12
A	WO, A1, 93/10 253 (ESCA GENETICS) 27 May 1993 (27.05.93), claim 1.	1
A	WO, A1, 92/13 961 (NIPPON STEEL CORPORATION) 20 August 1992 (20.08.92), claims 1,3-5,10,11.	1,12,14
A	US, A, 5 019 504 (CHRISTEN et al.) 28 May 1991 (28.05.91), abstract.	1

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

23 September 1994 (23.09.94)

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 94/00088

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